

Nanomechanical and thermophoretic analyses of the nucleotide-dependent interactions between the AAA⁺ subunits of magnesium chelatase

Nathan B. P. Adams*, Cvetelin Vasilev, Amanda A. Brindley and C. Neil Hunter

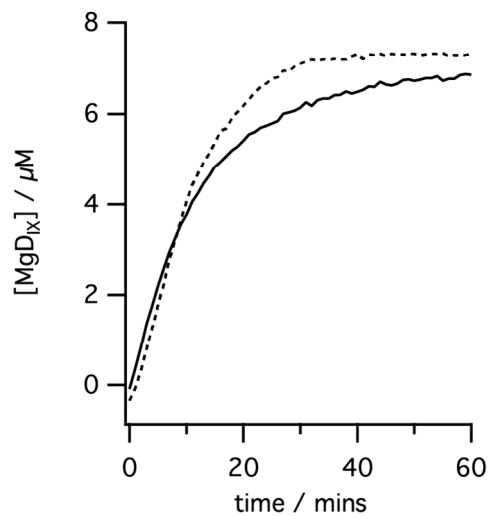
Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield S10 2TN, UK.

Supporting Information

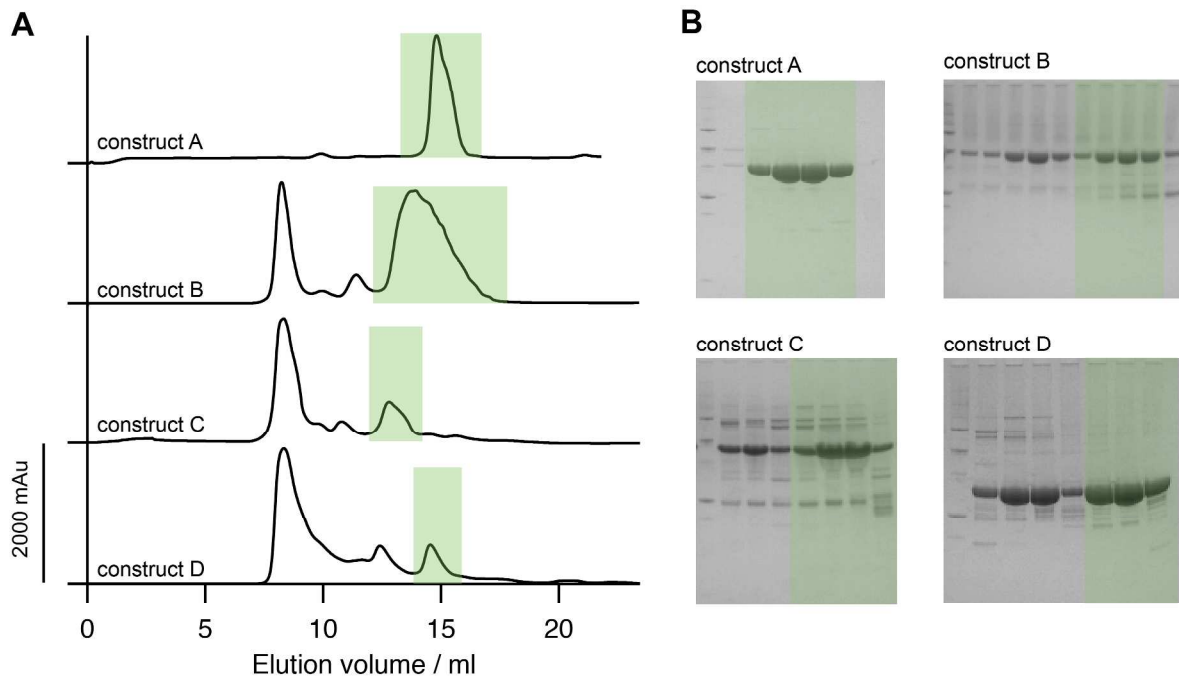
Supplementary Figures

ChlI	MTATLAAPSKTRRVVFPFTAIVGQDEMKLALLNVIDPKIGGVIM	60
ChlD	----MTTLTPFIPLNFPITAIVGQEAIKLALLLGAIDPGLGGIVIA	56
ChlI	ADLLPEIEVVANDPFNSSFSDPEMMSEEVRIRVDSQEPL---SIVKKKVTMVDLPLGATE	117
ChlD	HTLLPPIEIIKGNRYQCDPKNFGSWDDDTLEKFA-DVPLDQLETQVIPAPFIQIPLGVTE	115
ChlI	DRVCGTIDIEKALSEGKAFEPGLLAKANRGI	177
ChlD	DRLLGSVDVEKSVKQGEAVFQFGLLAQAHRGV	175
ChlI	EREGISIRHPARFVLVGSNPEEGELRPQLD	237
ChlD	EREGMSFQHPQCPLLIATYNPEEGPLRRHLLD	235
ChlI	QNPFPFCDQYQTEQALQAKIVNAQNLLPQVTIDYDRVKVSEVCAELDVG	297
ChlD	DSPISFIDQYDAELDDLKTTIILAREWLKEVSLTPEQVSYLEEATIRGGLQGH	295
ChlI	RAAKALAAFEGRTEVTVDISRVIVLCRLRHL--RKDPLE-----SIDSGSKV	343
ChlD	RVAKAIAALDGRSDVQADDLRQAVELVIV	355
ChlI	EKVFKR-VFGVVDE---A-----	357
ChlD	QEDQQDDKEDDKNEPEAEQDPSPISPEEFIFDPEGVSLDPSVLYFAQMAQKQKSGSRSV	415
ChlI	-----	357
ChlD	IFSDDRGRYLKPIPLPKGVRRIAVDATLRAASPYQKSRRLRHPDRQVIVEQGDIRGKKLV	475
ChlI	-----	357
ChlD	RKAGALIVFLVDASGSMALNRMQAAGAVMQLLTEAYENRDQVSLIPFQGENAEVLLPPT	535
ChlI	-----	357
ChlD	RSIAMAKKRLETLPCEGGSPLSHGLMQAVNVGMNAKSGDIGQVVIVAITDGRGNIPLAR	595
ChlI	-----	357
ChlD	SLGDEIPEGEKPDIAELLEIAAKIRGLGMQLLVINTEKKFVSTGFGKELAQAAGGKYYQ	655
ChlI	-----	357
ChlD	LPKATDQGIASMARQAIDMQ	676

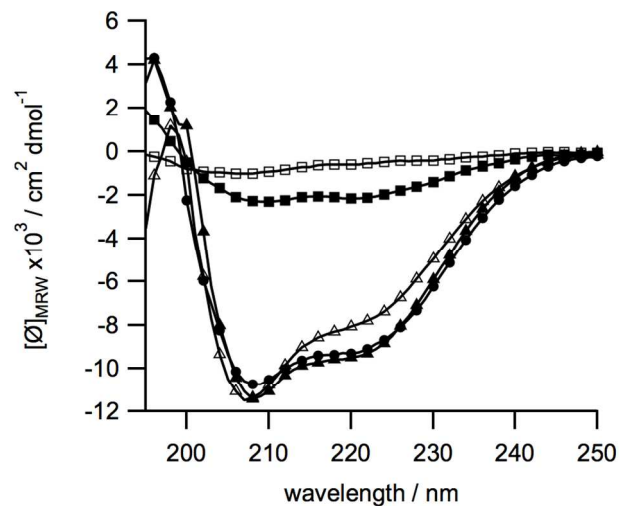
Supplementary Figure 1. Sequence alignment of ChlI and ChlD. Regions highlighted show the conserved AAA+ domains of the two proteins: Red, Walker A nucleotide binding motif; yellow, Walker B; green; arginine finger; blue, sensor II arginine. Black text – AAA+ domain, blue text – poly proline region and orange text – integrin I domain. Alignment performed using the Clustal W program.¹



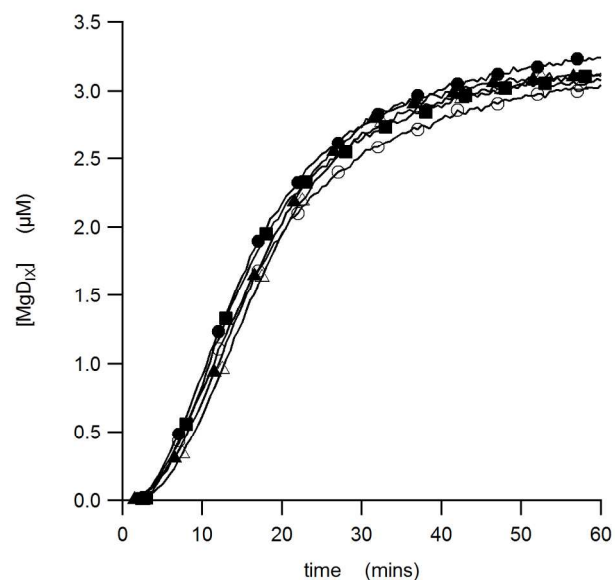
Supplementary Figure 2. Chelatase assays showing activity of ChlI C244S after labeling. ChlI cysteine mutants were assayed before and after labeling. Solid traces before labeling and dashes after labeling with Atto-488. Assays were performed in 50 mM MOPS/KOH, 0.3 M Glycerol, 1 mM DTT, 5 mM ATP, 15 mM MgCl₂, 8 μM DIX pH 7.7 34 °C, with 0.1 μM ChlD, 0.2 μM ChlI C244S and 0.4 μM ChlH. Protein concentration after labeling was estimated based on column efficiency.



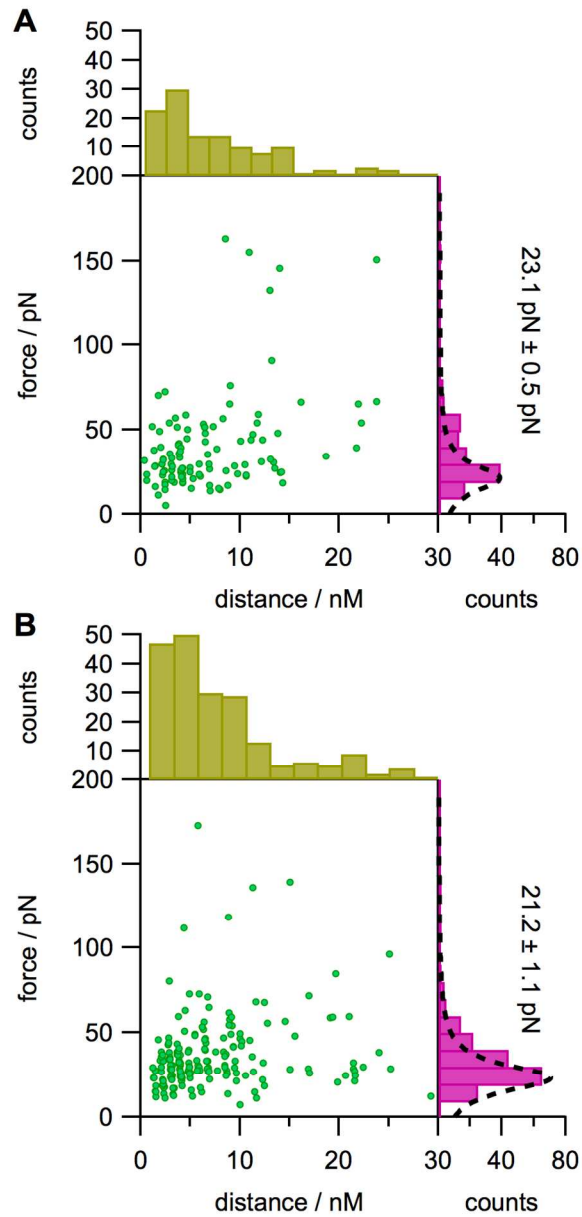
Supplementary Figure 3. **A**, gel filtration chromatography of truncations of ChID. Proteins were eluted with an isocratic gradient in 50 mM Tricine/NaOH, 200 mM NaCl, 0.3 M glycerol, 1 mM DTT, pH 7.9. Green shaded fractions were pooled and used for further experiments. **B**, 12 % SDS PAGE of proteins. Green shaded lanes correlate with shaded bands in **A**.



Supplementary Figure 4. CD spectroscopy (mean residue ellipticity) of artificial truncations of ChID (0.1 mg ml⁻¹) at 25 °C in sodium phosphate buffer, 1 mM β-mercaptoethanol, pH 7.4. Traces show wild type (●), truncation A (▲), truncation B (△), truncation C (□) truncation D (■).



Supplementary Figure 5. Progress curves of magnesium chelatase showing the ChIIIDH proteins catalyzing the formation of MgD_{IX} at 34 °C, in 50 mM MOPS/KOH pH 7.7, 0.3 M glycerol, $I = 0.1$, 5 mM MgATP^{2-} , 10 mM Mg^{2+} , 8 μM D_{IX} . ■, standard subunits non-tagged ChII, N-His₆-ChID; ●, N-His₆-ChII, N-His₆-ChII; ○, C-His₆-ChII, N-His₆-ChII; ▲, N-His₆-ChII, C-His₆-ChII; △, C-His₆-ChII, C-His₆-ChII. In all cases assays contained 0.1 μM ChID, 0.2 μM ChII and 0.4 μM N-His₆-ChIH.



Supplementary Figure 6. AFM Controls. **A**, 'clean' surface, **B**, BSA adsorbed surface. ChID was attached to a tip via its C-terminal His₆ tag. Experiments were performed in 50 mM MOPS/KOH, pH 7.7. **A** clean surface probability of interaction = 16 % with a fitted force of 21.2 \pm 1.1 pN, **B** BSA surface probability of interaction= 20.2 % with a fitted force of 23.1 \pm 0.5 pN.

Methods

Cloning of C-terminally *Synechocystis* tagged proteins

C-terminally His tagged proteins were produced by amplification of the ChlI and ChlD genes by PCR using Accuzyme (Bioline) according to the manufacturer's protocol. Primers used were, for ChlI:

ATTCCATATGACTGCCACCCTTGC and ATTCCTCGAGAGCTTCATCGACAACGCCAAAAACC,

for ChlD:

CCATATGACCACCCTGACCCCCTTTATTCC and CCTCGAGTTGCATGTCGGCGATCGCCTGG.

PCR products were digested with NdeI and XhoI and then ligated into pET21a (Novagen) digested with NdeI and XhoI. The resultant constructs were verified by sequencing (GATC Biotech, London). N-terminally tagged pET9a-His6 *Synechocystis* constructs have been described previously.² The arginine finger mutant of ChlD (R289A) was made using the QuikChange method (Agilent, Cheshire, UK) using the manufacturer's protocol.

Purification of proteins

Individual *Synechocystis* proteins His tagged protein containing cells were suspended in 25 mM Tris/HCl pH 7.4, 0.3 M glycerol, 0.5 M NaCl, 5 mM imidazole. Non His tagged ChlI cells were suspended in 50 mM Tricine/NaOH, 0.3 M Glycerol, 1 mM DTT, pH 7.9. Cell suspensions were sonicated and debris removed at 45 000 x g for 15 minutes at 4 °C. All His tagged subunits were purified using immobilised metal affinity chromatography on 5 ml Ni-chelating Sepharose columns (GE Healthcare). Bound proteins were washed with 20 mM imidazole and then eluted into 25 mM Tris/HCl pH 7.4, 0.3 M glycerol, 0.1 M NaCl, 400 mM imidazole.

All proteins were subject to further purification on a 1 ml Resource Q column (GE Healthcare). Protein was bound to the column in 50 mM Tricine/NaOH, 0.3 M Glycerol, 1 mM DTT, pH 7.9 and eluted by a 40 column volume linear gradient of NaCl to 1 M. A final polishing stage, to remove aggregated material, all proteins were subject to gel filtration on a 24 mL Superdex 200 Increase column (GE Healthcare) in 50 mM Tricine/NaOH, 200 mM NaCl, 0.3 M glycerol, 1 mM DTT, pH 7.9. Eluted protein was analysed by SDS-PAGE and monomeric protein pooled and stored at -80 °C.

Circular Dichroism spectroscopy

Spectra were recorded JASCO-810 spectrometer (JASCO, Great Dunmow, UK). Protein (0.1 mg ml⁻¹) was in 5 mM sodium phosphate, 1 mM β-mercaptoethanol, pH 7.4. Spectra were recorded stepwise from 250 – 200 nm (1 nm increments, 4 secs nm⁻¹, 4 scans) in a cuvette with a 0.2 cm path length, samples were in a cuvette with a 0.2 cm path length. Ellipticity was converted to mean residue ellipticity using the software included with the instrument. Thermal stability was determined by monitoring the ellipticity at 222 nm while increasing temperature.

Atomic Force Microscopy

Preparation of the sample substrates

Silica wafers were first cleaned in Piranha solution (3:1 concentrated sulfuric acid and 30 % hydrogen peroxide) for 1 hour, rinsed with milliQ water and modified as appropriate for the experiment.

Surfaces with adsorbed bovine serum albumin were immersed in a 5 mg ml⁻¹ solution of BSA in buffer A2 (50 mM Tricine/NaOH, 0.3 M Glycerol, 150 mM NaCl, pH 7.9) for 15 minutes before being stored in buffer A2.

For specific protein attachment an organosilane self-assembled monolayer (SAM) was assembled on the surface by chemical vapor deposition method. Briefly, the clean surfaces were placed into a glass desiccator purged with pure nitrogen for 10 min and then 20 µl of (3-aminopropyl)triethoxysilane (APTES, Sigma-Aldrich) was introduced into the desiccator. After another 5 min purge, the desiccator was evacuated down to a pressure of approximately 0.3 kPa by using a dry mechanical pump, and then sealed for 16 h to facilitate the deposition of the SAM. The next step in the surface functionalization, immediately after the SAM formation, was to attach a Ni-NTA functionality to the primary amines of the monolayer. This was done by using a homobifunctional crosslinker dimethyl suberimidate (DMS, Thermo Fisher Scientific) to attach N_α,N_α-Bis(carboxymethyl)-L-lysine (AB-NTA, Sigma-Aldrich) molecules to the monolayer, both chemicals were used at a concentration of 20 mM in PBS pH 7.4. After charging the NTA groups with 70 mM NiSO₄, the surfaces were incubated with ~800 nM of His-tagged ChlI in buffer A (50 mM Tricine/NaOH, 0.3 M Glycerol, 150 mM NaCl, pH 7.9), rinsed 3 times in buffer A and stored in the same buffer at 4 °C.

Preparation of functionalized AFM probes for directed ChlD attachment.

Hybrid AFM probes, Si tips mounted on Si₃N₄ triangular cantilevers, model SNL (Bruker) were first cleaned Piranha solution for 1 hour and rinsed with milliQ water. Immediately after that a SAM of (3-Mercaptopropyl)trimethoxysilane (MPTMS, Sigma-Aldrich) was assembled onto the AFM tips following the same method as for the sample substrates. Next, an amine-to-sulfhydryl heterobifunctional cross-linker with a 9.5 nm long polyethylene-glycol (PEG) spacer arm, terminated at one end with N-hydroxysuccinimide (NHS) ester group and maleimide (MAL) group at the other (SM(PEG)₂₄, Thermo Fisher Scientific) in order to attach AB-NTA molecules to the AFM probe. Both, the amine-targeted and thiol-targeted reactions were accomplished simultaneously in PBS pH 7.4 at a final SM(PEG)₂₄ concentration of 1 mM and a final AB-NTA concentration of 20 mM for 40 min. Then, the AFM probes were gently washed (4 times) in PBS pH 7.4 and stored in the same buffer for further use. In order to attach the ChID molecules the probes were charged with 70 mM NiSO₄ and then incubated with ~50 nM of the appropriate His-tagged ChID in buffer A, rinsed 3 times in buffer A and stored in the same buffer at 4 C.

The spring constants of the AFM cantilevers used were individually determined to accurately quantify the force data and varied between 0.19 and 0.23 Nm⁻¹. For the SMFS measurements the tip-sample contact force was kept in the range around 100 pN, the ramp repetition rate was 1 Hz and the ramp distance was 580 nm in order to fully stretch the flexible linker molecule on the AFM tip and to separate the ChID from the ChII molecules during each ramp cycle.

Subsequent image analysis was performed using Bruker Nanoscope Analysis v1.42 and OriginPro 2015 (OriginLab Corp.) software. Data reduction (positive identification of specific rupture events) was based on the analysis of the rupture events with tip-sample separations in the range 3 – 30 nm. The most probable values for the rupture force and the rupture length were obtained from the maximum of the Gaussian fit to the force and rupture length distribution combined in a statistical histogram.

Labeling ChII

ChII mutant C244S was desalted into 20 mM NaH₂PO₄, 150 mM NaCl, 3 mM ADP, 13 mM MgCl₂, pH 7.4 via a Zeba Spin column (Thermo Scientific) following the manufactures instructions and mixed with 1.3 fold excess of Alexa Fluor 488 C5-Maleimide (Life Technologies) for 2 hours while protected from the light. The reaction was quenched by the addition of 10 mM DTT for 10 minutes before desalting into 50 mM Tricine/NaOH, 0.3 M Glycerol, 200 mM NaCl, pH 7.9 via a PD-10 column (GE Healthcare). Protein was aliquoted and snap frozen in liquid nitrogen.

Microscale Thermophoresis (MST)

MST was performed using a NT.115 Monolith instrument (Nanotemper Technologies, Munich, Germany) using a Blue LED for excitation. The instrument settings were: 80 % blue LED excitation power, 40 % IR laser power. All samples were performed in a similar buffer (50 mM Tris/NaOH, 10 mM MgCl₂, 0.1 % Pluronic F127, 1 mg ml⁻¹ BSA, pH 7.8 at 20 °C in Premium MST capillaries. Baseline fluorescence was recorded for 5 seconds before inducing thermophoresis with an IR laser. MST traces were analyzed by taking the average fluorescence (F_{cold}) after the temperature jump on heating and after thermophoresis was observed (F_{hot}). Cold start 6.04 s, length 0.3 s; hot start 13.26 s, hot length 1.90 s. The ratio $\frac{F_{hot}}{F_{cold}}$ was plotted as a function of concentration of binding partner (e.g. ChID WT). The data was fitted to equation 1, where I is the concentration of labelled protein, c the concentration of titrant and K_d the calculated dissociation constant. using the supplied instrument software (NT Analysis, v 1.5.41) and K_d reported, and the fit parameters (bound and unbound) used to normalize the data to produce the 'fraction bound' for comparison to other binding partners, data was plotted using Igor Pro (v 6.35A, Wavemetrics, Oregon).

$$f(c) = \text{unbound} + \frac{(\text{bound} - \text{unbound})}{2 \cdot ([I] + [c] + K_d) - \sqrt{([I] + [c] + K_d)^2 - 4 \cdot [I] \cdot [c]}}$$

Equation 1 Binding equation for MST analysis.

Magnesium chelatase assays

Assays were performed in 50 mM MOPS/KOH, 0.3 M glycerol, 15 mM MgCl₂, 5 mM ATP, 8 μM DIX, $I = 0.1$ (KCl) at 34 °C. Assays were initiated by the addition of enzyme to give final concentrations of 0.1 mM ChID, mM 0.2 mM ChII and 0.4 mM ChIH. Steady state assays of magnesium chelatase followed formation of the product, MgDIX, over a period of one to two hours. Reaction progress was monitored using a Omega FluoStar microplate reader (BMG LabTech, Aylesbury, UK), with excitation at 420 ± 5 nm and emission detected at 580 ± 5 nm. Steady state rates (v_{ss}) were calculated using software supplied by the manufacturer (MARS version 2.41).

References

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M.; Wilm, a; Lopez, R.; Thompson, J. D.; Gibson, T. J.; Higgins, D. G. *Bioinformatics* **2007**, 23, 2947–2948.

(2) Jensen, P. E.; Gibson, L. C.; Henningsen, K. W.; Hunter, C. N. *J. Biol. Chem.* **1996**, 271, 16662–16667.